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The effect of fasting or calorie restriction on autophagy induction: a review of the literature

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Short running head: Fasting and autophagy

Highlights

- Both fasting and CR have a role in the upregulation of autophagy, the evidence overwhelmingly suggesting that autophagy is induced in a wide variety of tissues and organs in response to food deprivation.

Abstract

Autophagy is a lysosomal degradation process and protective housekeeping mechanism to eliminate damaged organelles, long-lived misfolded proteins and invading pathogens. Autophagy functions to recycle building blocks and energy for cellular renovation and homeostasis, allowing cells to adapt to stress. Modulation of autophagy is a potential therapeutic target for a diverse range of diseases, including metabolic conditions, neurodegenerative diseases, cancers and infectious diseases. Traditionally, food deprivation and calorie restriction (CR) have been considered to slow aging and increase longevity. Since autophagy inhibition attenuates the anti-aging effects of CR, it has been proposed that autophagy plays a substantive role in CR-mediated longevity. Among several stress stimuli inducers of autophagy, fasting and CR are the most potent non-genetic autophagy stimulators, and lack the undesirable side effects associated with alternative interventions. Despite the importance of autophagy, the evidence connecting fasting or CR with autophagy promotion has not previously been reviewed. Therefore, our objective was to weigh the evidence relating the effect of CR or fasting on autophagy promotion. We conclude that both fasting and CR have a role in the upregulation of autophagy, the evidence overwhelmingly suggesting that autophagy is induced in a wide variety of tissues and organs in response to food deprivation.

Abbreviations. Atg: autophagy-related genes. LC3: microtubule-associated protein 1 light chain 3. Sirt1: silent mating type information regulation 1. LAMP2: lysosomal-associated membrane protein 2. ULK1: Unc-51-like Kinase-1. AMPK: AMP-activated protein kinase. mTOR: mammalian target of rapamycin.

Keywords: Calorie restriction; Fasting; Autophagy

Autophagy

Autophagy literally means "self-eating" and is a vital self-degradative cleanup process that facilitates the removal of misfolded or aggregated proteins, as well as recycling of damaged cell components (1-5). In addition to intracellular aggregates and damaged organelle elimination, cellular senescence and cell surface antigen presentation could be promoted by autophagy (5). Moreover, autophagy has a protective role against genome instability and prevents necrosis (5). As a consequence, autophagy likely plays a substantial role in the prevention of a wide variety of diseases including cardiomyopathy, diabetes, liver disease, cancer, neurodegeneration, autoimmune disease and infections (5) and, conversely, deregulated autophagy is known to be associated with several disorders, including metabolic diseases, neurodegenerative disorders, infectious diseases and cancer (6).

Three primary types of autophagy are recognized: macro-autophagy, micro-autophagy, and chaperone-mediated autophagy. However, the term "autophagy" usually refers to macro-autophagy, which is the most prevalent form (5, 7-9). Although these types of autophagy differ morphologically, lysosomes play a crucial role in degradation and recycling in all of them (7). Macro-autophagy facilitates eradication of damaged cell organelles or unused proteins. In this regard, the material that needs to be degraded is engulfed by the phagophore, which forms a double membrane known as an autophagosome around the organelle marked for destruction (10, 11). Then, the autophagosome translocates to the lysosome and the two organelles fuse. Within the lysosome, acidic lysosomal hydrolases cause degradation of the contents of the autophagosome (5, 10). By contrast, in micro-autophagy, the lysosome itself directly takes up the cytosolic components via lysosomal membrane invagination (5, 7, 8). In chaperone-mediated autophagy (CMA), a very complex and specific pathway, the lysosomal membrane receptor lysosomal-associated membrane protein 2A (LAMP-2A) recognizes chaperone proteins (such as Hsc-70) which are accompanied by targeted proteins, leading to translocation

of the protein across the lysosomal membrane and subsequent unfolding and degradation (3-5). Degradation of damaged cellular components, such as mitochondria, through macro-autophagy seems to play a substantial role in cell protection from further oxidative damage, cellular dysfunction, and ultimately cell death (12). The autophagy that occurs under normal physiological conditions (basic autophagy) is indispensable for maintenance of cellular homeostasis and the balance between macromolecule synthesis and degradation (9).

Various physiological processes, such as neurolamine synthesis in dopaminergic neurons, surfactant biogenesis in pneumocytes, or erythrocyte maturation, are mediated through autophagy (9). Autophagy is also stimulated by various stress conditions (induced autophagy) such as oxidative stress (with the appearance of reactive oxygen species), unfolded proteins, viral infection or starvation (9). Autophagy promotion is crucial when the cell is deprived of compounds necessary for the synthesis of new molecules, in order to adapt to new and unfavorable conditions, as well as, ultimately, for survival under stress conditions (9). Autophagy also plays a salient role in protection of an infected cell from multiplication of viruses or bacteria (13, 14).

Despite the numerous beneficial effects of autophagy, long-term or overly activated autophagy could be harmful for cells and may result in cell death (15, 16), referred to as programmed cell death (apoptosis), type II or autophagy-associated apoptosis (9). In contrast to programmed cell death, type I (classical apoptosis) cell death, which is dependent on caspase enzymes, type II is dependent upon increased activity of lysosomal enzymes (9).

Nevertheless, halting autophagy for an extended period is detrimental to cells. For example, tumorigenesis may occur due to disturbances in cell growth and genome instability as a consequence of autophagy inhibition. By contrast, tumor cells are able to survive despite hypoxic conditions and nutrient deficiency, as well as during chemotherapy, due to autophagy induction (9). Autophagy dysfunction is associated with a variety of diseases, including cancer,

neurodegeneration, cardiovascular disorders, and microbe infection; this is because, for cell survival and function, it is necessary that unneeded or damaged cellular or non-self-components are sequestrated and efficiently cleared (17, 18). In conclusion, autophagy is crucial for cell health, and both over- and under-activity are deleterious to the cell.

Molecular Machinery of Autophagy

As the process and molecular machinery of autophagy has been previously described in some detail (5, 9), here we only touch upon these issues. Genetic screening has been extensively performed in yeast showing that 32 different autophagy-related genes (Atg) exist (notably, many of these genes are conserved in slime mould, plants, worms, flies and mammals, indicating that, in response to starvation, the autophagic processes are important across phylogeny) (5). In autophagy, cytosolic constituents, including proteins and organelles are sequestrated in the autophagosomes. Fusion of autophagosomes and lysosomes results in autolysosome formation (5). In mammals, autophagy is regulated by two kinases: protein kinases mTOR and AMPK. These proteins inhibit phosphorylation of the Unc-51-like kinases ULK1 and ULK2 (mammalian homologues of Atg1) (19, 20). The autophagy inhibitory pathway involves class I phosphoinositide 3-kinase (PI3K), the mammalian target of rapamycin (mTOR), and the pathway is well characterized. Autophagy is promoted via phosphorylation of Ulk1 by AMPK, although mTOR represses the process (4).

To control autophagy, the autophagic signaling pathway driven by autophagy-related genes (Atgs) is employed. Beclin1 (Atg6) and class III PI3K play a crucial role in the vesicle (or isolation membrane) nucleation step of autophagy, which is the initial step of autophagosome formation. The next step begins when Beclin-1 acts as a mediator to involve the other Atg proteins in the Class III PI3K complex. Two conjugation systems are involved in the vesicle elongation process. Conjugation of Atg12 to Atg5, with the help of Atg7 and Atg10, is the first pathway (4, 21). In the second pathway, phosphatidylethanolamine

conjugates to Atg8 (microtubule-associated protein 1 light chain 3; LC3) by the sequential action of Atg4, Atg7 and Atg3. In the latter process, the soluble form of LC3 (LC3-I) converts to the autophagic vesicle-associated form (LC3-II), an intrinsic autophagosomal membrane marker in autophagy (4, 21).

Fasting or calorie restriction

A variety of stress stimuli induce autophagy, including nutrient and energy stress, endoplasmic reticulum (ER) stress, pathogen-associated and danger-associated molecular patterns, hypoxia, redox stress, and mitochondrial damage (22). Fasting and CR are considered as an optimal intervention for improving health and lifespan, increasing resistance to stress, slowing aging and increasing longevity without the undesirable side effects associated with alternative interventions (23-25). CR, generally defined as a 10–40% reduction in caloric intake without a reduction in dietary nutritional content, exerts preventive effects on conditions such as cancer, hypertension, diabetes, and other age-related diseases in a wide range of animals, including humans and non-human primates (26, 27).

Beneficial effects on various physiological processes that are known to deteriorate with age have been attributed to CR which appears to increase longevity through various mechanisms, including changes in energy production and utilization, oxidative stress, insulin sensitivity, inflammatory responses, and alterations in the communication between cells and organs (Figure 1) (24, 25). The anti-aging effects of CR are attenuated when autophagy is inhibited (28); therefore, a growing body of evidence suggests that autophagy has a substantial role in CR-mediated longevity (29). CR represents the most robust non-genetic autophagic inducer, nutrient depletion or limitation being associated with lifespan-extension in many species (22). However, to our knowledge, no study has addressed the impact of CR or fasting on stimulation of autophagy. Thus, the main objective of the current study was to review the evidence linking CR or fasting with autophagy promotion.

The effect of fasting or calorie restriction on neuronal autophagy

A growing body of evidence indicates that autophagy is a key mechanism in the prevention of malignancy, infection and neurodegenerative diseases, as well as in slowing ageing (28, 30-34). The dependence of neurons on mechanisms of protein clearance for survival increases with age (35) as, in later life, protein accumulation reflects both the aging process and disease-related deficits, which hamper the elimination of damaged proteins that are contributory factors in neurodegenerative diseases (35-38). A reduction in proteostasis and formation of protein aggregates contributing to proteotoxicity and neuronal cell death is a common feature of neurodegeneration and aging (39, 40). To protect against neuronal proteotoxicity, autophagy eliminates the deleterious proteins from neurons (41, 42). Indeed, to prevent neuronal protein aggregation, macroautophagy is crucial (43-45), although constitutive autophagy is also critical in neurons for cell survival as it prevents ubiquitinated proteins from accumulating. However, overactive or dysfunctional autophagy could result in neuronal cell death in disease states.

Modulated autophagy plays a vital role in neurons because it underpins protein clearance, and defects in this molecular pathway are responsible for the selective neuronal susceptibility seen in neurodegeneration (46). Autophagy dysfunction is documented in Alzheimer's disease, Huntington's disease, and Parkinson disease, and autophagy induction is one of the early stress response mechanisms involved in the process of axonal dystrophy (46-53). It is postulated that neurodegenerative diseases and aging may be due primarily to the abrogation of autophagy in neurons, thus precipitating loss of proteostasis and leading to neuronal cell death (30-32, 46). Therefore, identifying a novel approach or developing a new drug to upregulate neural autophagy in the intact CNS has attracted considerable attention, and calorie restriction (CR) and food deprivation (fasting) offer a simple, safe and inexpensive alternative approach to induce autophagy.

As the brain is metabolically privileged (54, 55), it was previously believed that fasting or CR might not effect an upregulation of neural autophagy in the brain. However, Alirezaei et al showed that short-term fasting caused a dramatic up-regulation of neuronal autophagy in mice (56). In six- to seven-week old male C57BL/6J and GFP-LC3 (Tg/+) mice deprived of food, an upregulation of autophagy was seen after only 24 hours, and this effect was magnified after 48 hours in hepatocytes, cortical neurons and Purkinje cells in the cerebellum. In addition, an increase in GFP-LC3 occurred and the level of phospho-S6RP in the Purkinje cell bodies of food-restricted mice was dramatically reduced. Of note, phospho-S6RP levels indirectly indicate mTOR activity, and its activity is inversely associated with autophagy (56).

In another study, the effect of fasting on macroautophagy in neurons was assessed in an Alzheimer's disease (AD) mouse model (57); fasted three month old male mice showed an increase in the number, size and signal intensity of autophagosomes in neurons using time-lapse imaging; the autophagosome parameters were higher in the AD model mice before fasting, and increased more rapidly during fasting in comparison to the control mice. However, evaluation of the metabolism of exogenously labeled Aβ revealed that the activated macroautophagy was insufficient to degrade the intracellular Aβ increased by enhanced uptake from the extracellular space during fasting. Although intracellular accumulation of endogenous Aβ was increased by fasting, extracellular Aβ accumulation was not substantially decreased (57).

In a mouse model of Charcot–Marie–Tooth disease, fasted intermittently (IF) for five months (58), increased expression of autophagy-associated proteins, Atg7 and Microtubule-associated protein 1 light chain 3 (LC3) were found together with decreased levels of p62, a protein that serves as a link between LC3 and ubiquitinated substrates. Taken together, these findings indicate that autophagy increased in the nerves of IF neuropathic mice. Thus, the IF regimen stimulates the autophagy-lysosomal pathway in peripheral nerves (58). Notterpek et

al. evaluated the effect of CR on autophagy proteins within the peripheral nervous system, applying both *in vivo* and *in vitro* methods (59). For *in vitro* tests, Schwann cells from the sciatic nerves of young and old rats were isolated and the cells incubated in a medium deprived of amino acids and serum (Stv medium) to induce autophagy. In response to this nutrient deprivation, Atg7 and the conversion of LC3 I to LC3 II were increased in the cells from young (postnatal day 2) rats. However, in the cells from the older (25 months) rats, the response to Stv was reduced, especially for Atg7. In contrast to the cells from the older rats, in cells from the young rats the phosphorylated form of ribosomal protein S6 (pS6) completely disappeared in response to the Stv. Moreover, the ratio of pS6 and S6 was significantly decreased in cells from both young and old rats in response to the Stv, though the ratio was dramatically higher in cells from older versus younger rats, indicating defective autophagy in cells from the older rats. For *in vivo* assessment, male rats of different ages (8, 18, 29 and 38 months) were assigned to two groups: AL (ad libitum) or CR (calorie restriction) groups. Levels of the lysosome-associated membrane protein 1 (LAMP1) and the autophagic protein Atg7 gradually increased with age, indicating degenerative changes in peripheral nerves of aged animals. The CR intervention moderated this trend and decreased the age-associated increase in levels of pS6 and total S6, suggesting that CR muted the degenerative changes, and reduced the demand on protein homeostatic mechanisms, including the autophagy–lysosomal pathway (59).

A summary of the studies evaluating the effect of fasting or calorie restriction on neuronal autophagy is shown in Table 1.

The effect of fasting or calorie restriction on liver autophagy

The concept of autophagy was originally discovered after the identification of lysosomes in the liver in the 1960s (60, 61). Autophagy is induced in the liver due to the existing high levels of lysosomes and metabolic stress. Macronutrients, including amino acids, glucose and free fatty acids, which are released by liver autophagy can be used in energy

production and synthesis of new macromolecules for starved cells. In addition, the quality and quantity of organelles such as mitochondria can be controlled by liver autophagy (60). Liver autophagy is required to maintain liver homeostasis under normal physiological conditions, and for protecting the liver from stressors such as proteotoxicity, metabolic dysregulation, infection and carcinogenesis (61).

Recent evidence confirms the role of liver autophagy in basic hepatic functions, such as glycogenolysis, gluconeogenesis and β‑oxidation, which are mediated through elective turnover of specific cargos controlled by a series of transcription factors (60). Hepatic autophagy also plays a role in the prevention of liver diseases, derangement or malfunction of autophagy having a substantial role in the pathogenesis of liver diseases such as non-alcoholic fatty liver disease (NAFLD), nonalcoholic steatohepatitis (NASH), viral hepatitis and hepatocellular carcinoma (60-62). Lipid droplet degradation in hepatocytes (lipophagy) may be induced by autophagy, indicating the important role of autophagy in preventing NAFLD (63). By contrast, autophagy impairs cellular lipid accumulation, obesity and aging, all of which contribute to the pathogenesis of liver diseases. Therefore, augmentation of liver autophagy is a potential therapeutic approach in liver diseases (62).

Studies in older rats have shown that age-related lysosomal proteolysis in the liver is attenuated by fasting and calorie restriction (64-66), indicating the importance of autophagy in slowing the effects of aging. Donati et al assessed the effect of CR on the stimulation of autophagy in male Sprague–Dawley rats subjected to alternate day fasting, assessed by the rate of autophagic proteolysis in isolated rat liver cells *in vitro* (67). Maximum rates of autophagy were achieved in the CR group relative to controls at all ages (67).

Kovacs et al studied the effect of fasting on serum insulin and the size of the autophagic-lysosomal compartment in liver and exocrine pancreatic cells of the mouse (68). Male mice were fasted for 24 hours, then re-fed for 3 hours, after which periods of fasting were increased

(2, 12, 24, 48 and 72 hours) in the experimental groups. The lowest cytoplasmic volume fraction of autophagic vacuoles (AV) and dense bodies (DB) and the highest insulin levels were observed in both liver and exocrine pancreatic cells after 3 hours feeding. In comparison to the 3 hours feeding, the cytoplasmic volume fraction of AV significantly increased after 12, 24, 48 and 72 hours of fasting in both liver and exocrine pancreatic cells. Compared with 3 hours feeding, the cytoplasmic volume fraction of DB significantly increased after 12 hours of fasting and slightly increased after 72 hours of fasting in the liver. A significant increase in the cytoplasmic volume fraction of DB in the exocrine pancreatic cells was seen after 48 and 72 hours of fasting compared with after 3 hours of feeding. A gradual decrease in the level of serum insulin was observed throughout the whole fasting period (68).

Krustew et al. studied the impact of 1-8 days of fasting on the size and number of lysosomes in the liver cells of rats (69), and a notable increase in the number of lysosomes, particularly secondary ones, was observed. In addition, a direct association between autophagy and starvation was observed (69).

In a recent study by Luévano-Martínez et al, the effect of CR on induction of autophagy in liver mitochondrial membranes of mice was investigated (70). After four months of CR dietary intervention, a notable increase was found in the LC3-II/LC3-I ratio, indicating enhanced liver mitochondrial autophagy. The authors suggested that, since higher mitochondrial numbers were found in the CR group, dietary-induced modifications in mitochondrial numbers in the liver could not be explained by enhancing autophagy; however, activating autophagy and mitochondrial biogenesis simultaneously (which are both induced by CR) could enhance mitochondrial turnover and result in a healthier mitochondrial pool (70). The protective role of fasting against ischemia reperfusion (IR) injury in the liver of mice was investigated (71); the results showed that even one day of fasting is protective against hepatic IR injury and that an upregulation of LC3 II and beclin1 occurred, indicating enhanced

autophagy, together with a reduction in circulating high mobility group box 1 (HMGB1) which is associated with cytoplasmic HMGB1 translocation, aggregate formation, and autophagy. Both supplementation with HMGB1 or inhibition of autophagy re-elevated circulating HMGB1, thus abolishing the protective effect of fasting. Another finding was that one day fasting caused upregulation of hepatic Sirt1 activity, important because amongst the responses to starvation is induction of the autophagic response regulated by Sirt1. Moreover, the autophagy inhibitor SP600125 confirmed the protective role of autophagy, revealed an increase in ischemia reperfusion injury (IRI) and in serum HMGB1 both before and after IR in fasted mice, suggesting a central role for autophagy in the fasting-mediated HMGB1 reduction and protection. Thus, the protective effects of fasting were mediated via an HMGB1-dependent process regulated through Sirt1 and involving autophagy (71).

The effect of fasting on autophagy in the liver of α_1-antitrypsin (α_1-AT) deficient mice was evaluated by Teckman and colleagues (72). α_1-AT deficiency results in liver injury, with an intense autophagy response; using TEM, the hepatocytes of fasted (18 h) C57/B6 mice showed an increase in AV in the cytoplasm. By contrast, in the PiZ mouse liver, no difference in the number of autophagosomes was seen post fasting. No increase in hepatic fat accumulation was seen in either strain. In comparison to wild type (WT) mice, a decreased tolerance for prolonged fasting was evident in the PiZ mouse (72).

The effect of age and CR diet on the heart and liver of Fisher 344 rats was investigated by Wohlgemuth et al (12). The rats were assigned to four groups; 6 and 26 months rats, with each age category receiving either AL or CR diet. Age had no effect on cardiac fractional volume of AV; however, a 100% higher fractional AV volume was found in rats on CR diet. Neither age nor diet had any effect on fractional AV volume in the liver tissue. At the protein level, an increase in expression of beclin-1, LC3-II and procathepsin D was found with increasing age in heart tissue, while expression of LAMP-1 decreased with age. In addition,

the interaction between age and diet was affected by the expression of Atg7 and Atg9 and the ratio of LC3-II/LC3-I, in which a dramatic increase was observed in protein expression and LC3 ratio in CR animals, findings in line with the elevated autophagy response in heart tissue. In the liver, neither age nor an interaction between age and diet had any effect on expression of Atg7, LC3-II, and the LC3-II/I ratio. Likewise, diet did not cause a substantial effect on the expression of any of these proteins in the liver. Taken together, CR increased autophagy in the heart of rats, suggesting that CR may have a potential cardioprotective benefit by stimulating autophagy (12).

The effect of fasting on energy homeostasis of adipose triglyceride lipase-deficient (ATGLAKO) mice was investigated by Wei Wu et al (73). ATGLAKO animals were fed normally or food deprived for 5- and 48-h, and energy metabolism as well as changes in transcription of autophagy-related proteins evaluated. Raised LC3 mRNA (encoding autophagy essential protein (74)) was found in white adipose tissue (WAT) of ATGLAKO mice post-fasting, together with increased 14-kDa E2 and LC3 mRNA in the liver and an increase in the transcription of LC3 and of two ubiquitin ligases, Atg1 and muscle RING-finger protein-1 (MuRF-1), markers for ubiquitin-related degradation (75), in the muscle and heart of these mice (73).

Yamamoto et al. investigated the differential adaptive responses to fasting on various tissues of mice (76). Quantitative PCR was used to evaluate cellular responses in several tissues after one or two days of fasting. Post-fasting, the expression of LC3b and p62 (essential components in autophagosome-mediated macroautophagy) (77, 78), were upregulated; the expression of LC3b was induced most in the heart, followed by skeletal muscle, thymus, lung, small intestine, testis, colon, and liver, while the expression of p62 was again most increased in heart followed by skeletal muscle, thymus, colon, lung, kidney, spleen, and small intestine. Post-fasting, a significant increase in Lamp2 (a receptor for substrate proteins of chaperone-

mediated autophagy, which is involved in microautophagy (79)), was observed only in thymus (76).

In a recent study, the effect of fasting on induction of hormone fibroblast growth factor 21 (FGF21) in lysosome homeostasis was assessed (80). In comparison to fed mice, after 12 or 48 hours fasting, FGF21 levels as well as autophagy (reflected by increased LC3-II and decreased P62 levels) was found to be increased in the liver. This procedure was then repeated in 24-week-old wild-type (WT) mice and $Fgf21^{-/-}$ mice; after 24 hours of fasting, autophagy was increased in the hepatocytes (adjudged by the number of GFP-LC3 puncta, LC3-II turnover and the increased levels of P62) in WT mice. However, after 24 hours fasting, much higher levels of LC3-II and P62 were found in $Fgf21^{-/-}$ mice compared with WT animals, although similar levels of autophagy markers were observed in fed WT and $Fgf21^{-/-}$ mice, suggesting that in fasted $Fgf21^{-/-}$ mice the lysosomal-autophagic pathway is blocked. In addition, there were no differences in mTOR or AMPK activities in either WT or $Fgf21^{-/-}$ mice under feeding or fasting conditions. Overall, it appears that FGF21 does not have an effect on autophagy initiation, though may affect lysosomal function. Results of this study also showed that to regulate lysosomal function, transcription factor EB (TFEB) (which is considered to be a regulator of lysosome biogenesis and autophagy (81)) acts downstream of FGF21 signaling (80).

The effect of graded levels of CR on autophagy was investigated in mice using the hepatic transcriptome (82). Male mice were assigned to an AL diet or graded levels of CR (0% to 40% CR) for three months. Results demonstrated that autophagy was induced by starvation and nutrient deprivation. There was a positive correlation between downstream ATG genes (ATG genes control autophagosome formation), LC3, LC3-I and LC3-II and CR. Thus, a significant increase was found in autophagy levels with increasing levels of CR. In addition,

there was a negative correlation between the expression of genes involved in autophagy and circulating levels of insulin like growth factor 1 (IGF-1) (82).

A summary of the studies evaluating the effect of fasting or calorie restriction on liver autophagy is shown in Table 2.

The effect of fasting or calorie restriction on heart autophagy

The role of autophagy in heart health has attracted considerable attention. It is not currently known whether autophagy has protective or harmful effects on human cardiomyopathies (83). However, accumulated evidence suggests that cardiac homeostasis and function are predominantly regulated by autophagy (84). In normal physiologic conditions, autophagy is important for conservation of cardiac structure and function (84). Like other tissues, autophagy in the heart mediates the synthesis of essential proteins during starvation by providing nutrients during stress and limiting damage under most conditions such as starvation, which may result in increasing cell survival (84, 85). Modulated autophagy is crucial to reduce cardiac injury and preserve cardiac function during heart ischemia (84). Cardiac adaptation is mediated by autophagy in pressure overload by restricting misfolded protein accumulation, mitochondrial dysfunction, and oxidative stress (84). Aging-induced cardiac abnormalities, development of cardiac proteinopathy and dysfunction, doxorubicin-induced cardiomyopathy and heart failure could all occur as a consequence of autophagy impairment (84).

Nevertheless, excessive autophagy is maladaptive, leading to cell death, and is involved in the pathogenesis of several heart abnormalities, contributing to cardiac proteinopathy, doxorubicin-induced cardiomyopathy, heart hypertrophy and failure (84-88). Indeed, autophagy activation can occur through some mediator proteins, such as AMP-activated protein kinase (AMPK), which are activated during myocardial ischemia when the heart is starved, and could, in fact, be beneficial; elevated expression of some other mediator proteins,

such as beclin 1, which occurs during restoration of blood flow (reperfusion), may be detrimental for the heart (4, 89, 90).

Modulated autophagy is now recognized as necessary for maintaining cellular energy homeostasis and is considered as an attractive and novel protective mechanism for the cardiovascular system, and as a new treatment for cardiac diseases (84, 87, 91). Nutrient deprivation, restriction of energy, lack of oxygen, absence of growth factors, and cellular stress are the main factors involved in the induction of autophagy (92, 93).

Several studies have investigated the effects of calorie restriction or food deprivation (fasting) on heart autophagy. To assess the efficacy of fasting on heart autophagy, male FBN rats were randomly divided into 6 groups (1) AL, (2) 20% CR, (3) 5 mg/kg/day resveratrol (Resv-5), (4) 50 mg/kg/day resveratrol (Resv-50), (5) CR with 5 mg/kg/day resveratrol (CR + Resv-5), and (6) CR with 50 mg/kg/day resveratrol (CR + Resv-50) for six weeks (94). All groups received equal amounts of proteins, vitamins, and minerals, while all three CR groups received 20% less food from a 125% fortified diet; rats in the AL group also received supplementation tablets but without resveratrol. To induce oxidative stress, a single intraperitoneal injection of 10 mg/kg doxorubicin (Sigma) or saline control was injected, and the expression of LC3B, p62, beclin-1, and Atg5–Atg12 conjugated in the left ventricle was assessed to show the level of autophagy induction. Results showed that the ratio of LC3-II/LC3-I did not change in the left ventricle in any of the intervention groups. In comparison to the AL group, a significant decrease was found in expression of p62 in CR and Resv-50 rats, indicating enhanced autophagy in the left ventricle of the animals in this group. Likewise, a notable increase was observed in the expression of beclin-1 in the left ventricle of the CR and Resv-50 animals compared with AL rats. However, there were no differences in Atg12–Atg5 levels (proteins that are linked to the growing phagophore) in the left- ventricular homogenates of rats in the intervention groups and AL rats. Treatment with doxorubicin increased the

frequency of apoptosis and creatine kinase levels (indicative of muscle damage) and lactate dehydrogenase (LDH) (an oxidoreductase enzyme); however, the enhanced autophagy due to CR + Resv-50 dramatically attenuated the doxorubicin-induced damage (94).

In another study, the effect of CR on autophagy was assessed in diabetic rat hearts (95). Diabetic and non-diabetic control rats were assigned to two groups: AL and CR (30% energy reduction) groups for 32 weeks. The LC3-II/LC3-I ratio was notably increased in the heart and liver of CR diabetic rats compared with AL diabetic animals. However, no significant change was found in the LC3-II/LC3-I ratio in the heart and liver of CR animals compared with AL non-diabetic rats. In addition, no significant difference was observed in the expression levels of beclin 1 among all the study groups (95).

Andres et al. investigated the signaling and autophagy responses to fasting in the hearts of obese mice (96). Male FVBN mice were divided into two groups, AL with normal chow (13% kcal/100 kcal fat) or a lard-based high-fat diet (fat 60% kcal/100 kcal fat) for 4–20 weeks. Then, the mice were subjected to overnight fasting. At the end of the study, in comparison to the age matched AL group, obesity, elevated fasting blood glucose, and insulin resistance developed in the mice in the high fat diet group. After 24 hours fasting, LC3-I conversion to LC3-II was significantly increased in lean mice, though no difference was found in diet induced obesity (DIO) mice. Autophagosome-lysosome fusion was then blocked using chloroquine to assess autophagic flux, indicating that LC3-II did not increase in DIO mice after lysosomal blockade, thus reflecting impaired autophagic flux. In addition, in response to fasting, an appropriate upregulation of mRNA for LC3B and p62/SQSTM1 was found, indicating that cardiac autophagy is blunted at the posttranscriptional level in DIO mice. Moreover, fasting increased phosphorylation of AMPK and enhanced dephosphorylation of S6 (indicating mTOR suppression) in both lean and DIO mice, while mTOR suppression was greater in DIO mice (despite the development of the metabolic syndrome), suggesting the intact induction of

autophagy at the posttranslational level which is controlled by the major energy- and nutrient-sensing pathways in the heart. Further analysis showed that protein networks involved in oxidative phosphorylation, autophagy, oxidative stress, protein homeostasis, and contractile machinery were altered under fed and fasted conditions in the hearts of lean and obese mice. Overall, results of this study demonstrated that fasting induces autophagy in the heart of lean mice, though impaired autophagy, an altered proteome, and a discordant response to nutrient deprivation occurred in the heart of obese animals and could potentially explain the increased injury associated with ischemia and reperfusion (96).

The effect of intermittent fasting on the autophagy-lysosome machinery in the myocardium was recently assessed by Godar et al (97). In this study, mice were fasted for 24 hours, followed by 24 hours re-feeding, or were fasted for 24 and 48 hours for six weeks compared with non-fasted age matched controls. Although no significant change was found in autophagosome abundance after 24 hours fasting, a dramatic increase was observed after 48 hours fasting. For inhibition of lysosome acidification and prevention of autophagosome processing, concomitant treatment with CQ (chloroquine) was applied, and indicated that, compared with the basal state in non-fasted mice, after 24 hours of fasting the abundance of LC3-II and SQSTM1/p62 (an autophagy substrate) were dramatically increased. However, following a fed day in the mice subjected to six weeks of every other day fasting, no change was found in the abundance of autophagosomes. In addition, in fed mice, no changes were seen in autophagic flux assessed with concomitant CQ treatment. Taken together, these results indicate that cardiomyocyte autophagy was induced by each episode of fasting, then returned to basal levels on the fed days. Moreover, in comparison to the non-fasted control mice, 24 hours fasting resulted in robust transcriptional induction followed by downregulation of the autophagy-lysosome genes in the myocardium. This was coupled with a notable accumulation

of TFEB protein in the nuclear subfraction after 24 hours fasting, which was followed by a rapid reduction after refeeding (97).

In another study, the potential role of forkhead transcription factors of O group 1 (FoxO1) in cellular signaling during CR was examined (98). Two groups of mice were studied, FoxO1-knockout heterozygous mice ($FoxO1^{+/-}$) and wild-type mice (WT). Mice in both groups were fed AL or 30 % CR. After 20 weeks intervention, a significant increase was found in the LC3-II/LC3-I ratio, while the expression of p62 was significantly decreased in the heart tissue of WT (of note, p62 accumulation shows impaired autophagy and its degradation indicates the activation of autophagy). However, no changes occurred in the LC3-II/LC3-I ratio or the expression of p62 in $FoxO1^{+/-}$. In addition, immunofluorescence staining of 8-OHdG (a marker of oxidative DNA damage) was performed to investigate oxidative DNA damage in the heart of animals. The results showed that, although the level of 8-OHdG was not changed in $FoxO1^{+/-}$ mice-CR, it was significantly decreased in WT-CR mice. These data suggest that autophagy increased in WT-CR mice, but its level did not change in $FoxO1^{+/-}$ mice-CR, demonstrating that FoxO1 may play a beneficial role in autophagy induction under conditions of CR (98).

A summary of the studies evaluating the effect of fasting or calorie restriction on heart autophagy is shown in Table 3.

The effect of fasting or calorie restriction on muscle autophagy

Skeletal muscle is the most abundant body tissue, comprising approximately 40% of the body weight, and is the major site of metabolic activity in the normal weight human. Muscle provides amino acids through protein breakdown, which can be used by other organs to produce energy during catabolic stress conditions (99). The autophagy-lysosome and the ubiquitin-proteasome are the major proteolytic systems in the human body which control skeletal muscle protein degradation (99). Although it has previously been hypothesized that myofiber atrophy is mediated by autophagy in the young, and that to prevent sarcopenia in the elderly autophagy

inhibition would be necessary, recent studies have shown that basal autophagy is crucial for muscle mass preservation and for maintenance of myofiber integrity and adaptation (99, 100).

Excessive autophagy contributes to muscle wasting and atrophy, through inhibited or altered autophagy, and can cause myofiber degeneration and muscle weakness (99, 101, 102). In this regard, a previous study has shown that deletion of a crucial autophagy gene in muscle, Atg7, is associated with muscle atrophy and the presence of protein aggregates, abnormal mitochondria, membrane body accumulation, sarcoplasmic reticulum distension, vacuolization, oxidative stress and apoptosis (103). In addition, muscle loss was increased during denervation and fasting in inhibited autophagy mice (103). It has been suggested that, to prevent sarcopenia, delay systemic aging, and extend health span, an appropriate induction of basal autophagy is essential and plays a salient role in promotion of the selective clearance of dysfunctional organelles and damaged proteins (100, 104).

Overall, it is now accepted that autophagy flux is indispensable for muscle health and prevention of muscle loss and weakness, by removing toxic proteins and dysfunctional organelles (99-102).

Stressor agents, such as exercise and muscle contraction and food deprivation, were previously introduced as autophagy stimulators in muscle (105, 106), and in this review we summarize the results of the studies evaluating the effect of CR or fasting on muscle autophagy. In one study, male Fischer-344 rats were assigned into four groups, three fasting groups (fasted for one, two or three days, respectively) and one control group (sedentary) (107). A duration-dependent increase was found in LC3-II in fast-twitch plantaris muscle after two or three days fasting, as well as in slow-twitch soleus muscle after three days though at a lower magnitude. In plantaris muscle, the expression of p62 was increased in all fasting groups, though in the soleus muscle p62 increased only after two days of fasting and with a lower magnitude than in plantaris muscle. In addition, the expression of total and phosphorylated Akt (the protein which

negatively impacts autophagy through activation of mTOR) was decreased in both muscles in response to fasting. No notable changes were found in total or phosphorylated mTOR in soleus after fasting, however, and although no differences were found in total mTOR protein in plantaris muscle, a significant downregulation was observed in the phosphorylated mTOR after one or three days of fasting. Moreover, fasting had no effect on Forkhead box O3a (FOXO3a) levels (downstream targets of Akt), in any of the groups regardless of the type of muscle (107).

In another study, the effect of antioxidant supplementation and fasting was assessed in skeletal muscle of mice (108). Male mice were randomly assigned to four groups: 1) a control group which received neither drug or fasting, 2) an intervention group which was fasted without drug, 3) a control group which received N-Acetylcysteine (NAC) as a known antioxidant in the body for three weeks (100mg/kg bodyweight/2 day) without fasting and 4) an intervention group which received NAC for three weeks (100mg/kg bodyweight/2 day) with fasting. The results showed that NAC supplementation significantly reduced mRNA expression of Atg6, Atg7, Atg8 and Atg9 in skeletal muscle. However, with the exception of Atg9, the Atgs were upregulated in response to fasting. In addition, the ratio of LC3 II/LC3 I was significantly increased in the skeletal muscle of the both fasting groups compared with control groups (108).

In a recent randomized clinical trial, Rittig et al, investigated the effect of anabolic leucine-rich whey protein, carbohydrate, and soy protein with and without β-hydroxy-β-methylbutyrate (HMB) following three days of fasting (as an inducer of catabolism) on cell signaling factors related to muscle autophagy (109). Eight males, with a mean age of 24 years and mean body mass index (BMI) of 25 kg/m^2, were randomly recruited into four groups to receive: 1) a beverage containing carbohydrate (CHO), 2) a beverage based on leucine-rich whey protein (LWH), 3) a beverage based on soy protein (SOY) and 4) a beverage based on soy protein +3 g HMB (HMB). Participants in all groups were fasted and only allowed to drink tap water AL for 36 hours prior to the start of the trial. Results indicated that phosphorylation

of mTOR, Akt (the activator of mTOR, mediating autophagy inhibition) as well as the downstream target of mTOR, 4E binding protein 1 (4EBP1), were elevated in all 4 groups during the sipping period compared with the fasting period. In comparison to the fasting period, the phosphorylation of the other downstream target of mTOR, S6, was increased during the sipping period in the LWH group. Furthermore, compared with the fasting period, a decrease was found in the LC3II/LC3I ratio during the sipping period for LWH and SOY (109).

Vendelbo et al investigated the effect of fasting on human skeletal muscle autophagy and the potential mechanisms involved in cell signaling to induce autophagy after fasting (110). Eight healthy male volunteers participated in the study, with a mean age of 26 years and BMI 23.8 kg/m^2, and without a family history of diabetes mellitus. Subjects were randomly divided into two groups: 1) 72 hour fast, drinking tap water AL with normal activities, 2) overnight fast of 10 hours (control group) for >1 month. At the completion of the study, forearm net phenylalanine release was significantly increased, and its disappearance rate was decreased by fasting. mTOR phosphorylation was reduced by about 40% in response to fasting. In addition, mTOR phosphorylation was stimulated by insulin; however, its increase was substantially reduced during fasting. The phosphorylation of the downstream targets of mTOR, 4EBP1 and UNK-51-like kinase 1 (ULK1), were significantly decreased after 72 hours fasting and (LC3)B-II protein content was increased by about 30% compared with LC3B-I. LC3B-II levels were reduced by insulin stimulation on both experimental days with no effects of fasting. A significant increase in p62 protein expression after fasting was found, and was unchanged by insulin stimulation. Both fasting and insulin stimulation had no effect on FOXO3a phosphorylation. The authors concluded that there was an association between net phenylalanine release in skeletal muscle and reduced mTOR activation, which caused a decrease in downstream signaling towards cell growth (110).

Wohlgemuth et al investigated the effect of age and lifelong mild CR on skeletal muscle (plantaris muscle) autophagy and lysosome-related proteins (111). Male Fischer 344 rats were randomly assigned to four groups, (1) young sedentary AL fed (Y-AL), (2) old sedentary AL fed (O-AL), (3) old life-long 8% CR (O-CR) and (4) old life-long 8% CR with life-long daily voluntary wheel running. Animals were sacrificed at either 6 or 24 months of age. Results indicated that the expression of Atg6, Beclin-1 (which contributes to pre-autophagosome structure induction and formation) did not change in the plantaris muscle of the animals in the CR and CR/exercise groups in comparison to the old controls. However, there was a positive association between Beclin-1 expression and age; the expression of Atg7 (a necessary protein for the formation and expansion of the autophagosome), was notably increased in O-CR and O-CREx compared with O-AL, though it was not affected by increasing age. CR treatment or age had no effect on Atg9 expression (required for formation of autophagosomes) while, in comparison to O-AL, a significant increase was found in O-CREx. Age and CR with or without exercise had no significant effect on LC3 gene expression or the ratio of LC3-II to LC3-I (the indicator of ongoing autophagy). Finally, the mRNA levels of lysosomal-associated membrane protein 2 (LAMP-2), the crucial protein for completion of the lysosomal–autophagic degradation process, were dramatically higher in O-CR and O-CREx animals compared with O-AL. Significantly lower LAMP-2 mRNA levels were observed in the plantaris muscle of O-AL rats in comparison to the Y-AL rats (111).

The effect of long-term CR on human skeletal muscle was investigated in a recent clinical trial study (112). In this study, a total of 111 volunteer adults were assigned into three groups (37 age and sex matched subjects per group). A CR diet with adequate nutrients for 6 ± 3 years (range 3–15 years) was consumed by the participants in the first group (CR group). Participants in the second group had run for an average of 48 miles/week for 21 ± 11 years (range 5–35 years) (EX group), and in the third group, participants followed sedentary behavior

(regular exercise < 1 hr/week) (WD group). The results showed that several autophagy genes, including ULK1, ATG101, beclin-1, APG12, LC3, GAPRAP/GATE-16, and autophagin-1, were significantly upregulated in response to CR. In comparison to the WD control subjects, a dramatic increase was found in beclin-1 and LC3 protein expression levels in the skeletal muscle of the CR volunteers. A higher level of serum cortisol was observed in the CR group than in age-matched sedentary and endurance athlete groups (and it is well recognized that glucocorticoids may contribute to autophagy induction in several cell types (113, 114)). Moreover, several inflammatory factors, including the transcription factor necrosis factor kB (NF-kB), signal transducer and activator of transcription 5 (STAT5) and c-FOS, and, downstream, the mRNA levels of multiple inflammatory cytokines, including TNF- α, interleukin-6 (IL-6), interleukin-8 (IL-8), and inducible nitric oxide synthases (iNOS) were significantly downregulated in response to the CR. A lower serum TNF-α was shown in the CR group compared with the WD group. Taken together, the results of this study demonstrate that lifelong CR can increase serum cortisol, decrease inflammation and increase autophagy in cells, resulting in an increase in cellular protein quality and a decrease in dysfunctional proteins and organelles (112).

A summary of the studies evaluating the effect of fasting or calorie restriction on muscle autophagy is shown in Table 4.

The effect of fasting or calorie restriction on kidney autophagy

Progressive postmaturational deterioration of tissues and organs arises as a result of increasing age, and causes functional impairment of tissue, increased vulnerability to stress, and death (115). One of the major target organs of age-associated tissue damage is the kidney and, with increasing age, the incidence of chronic kidney disease (CKD) increases (115). However, to date, the underlying mechanism of age-associated kidney damage is unclear. CR is known to have a positive effect on longevity and healthy aging, extending lifespan and

enhancing physiologic function in several species (116). CR leads to a delay, or even a reversal, of kidney aging markers (117, 118). Among several mechanisms which have been suggested to explain the beneficial effects of CR on lifespan prolongation and healthy aging, increased autophagy activity and decreased dysfunctional mitochondria are the most important (119). Autophagy deregulation is associated with several renal disorders, many of them directly related to aging (120).

To assess the effect of CR on autophagy in aged mouse kidney, six-week-old mice were provided food and water AL for 12 months (115). Then, mice were assigned to 2 groups to receive food AL or a 60% AL diet (CR group) for the following 12 months. Results showed normal mitochondrial morphology with numerous auto(lyso)phagosomes in proximal tubular cells (PTCs) of CR mice in comparison to the control group. In addition, higher levels of sequestosome 1 (Sqstm1), (a marker for in vivo impaired autophagy) were found in the kidney of AL mice. The ratio of LC3I to LC3II conversion and LC3 dots (a marker of enhanced autophagy) were higher in the CR mice, indicating age-dependent mitochondrial oxidative damage in the kidney might occur as a consequence of impaired autophagy, and CR-mediated mitochondrial protection could be explained by enhancement of autophagy (115).

The effect of CR on renal autophagic activity of aged rats was investigated by Ning et al (119). 25 month old rats were divided into 2 groups: Old AL (OLA) group and OLD CR (OCR) group (in which the amount of food was 40% restricted compared with the AL group) for eight weeks. At the end of the study, in addition to the 27 month old rats, 3 month old rats were sacrificed (YAL) as a control group. In comparison to the young rats, the ratio of LC3-II/LC3-I in the kidney was decreased in the old animals. However, CR markedly increased the renal LC3-II/LC3-I ratio in the old rats compared with OAL. Expression of Beclin-1 was increased in the kidney of old animals and it increased significantly more in OCR versus OAL. Moreover, the p62/SQSTM1 expression and polyubiquitin aggregates were significantly

increased in OAL, and CR decreased them in aged kidney. These results indicate that in the aged kidney of rats, autophagy flux decreased and, importantly, that CR can improve it (119).

A summary of the studies evaluating the effect of fasting or calorie restriction on kidney autophagy is shown in Table 5.

The effect of fasting or calorie restriction on pancreas autophagy

Increasing age causes a reduction in glucose metabolism leading to glucose intolerance and a high prevalence of type 2 diabetes (T2DM) in the elderly population (121). Visceral adiposity generally increases and insulin sensitivity decreases among elderly individuals, further stressing pancreatic beta cells and increasing the risk of diabetes mellitus (122, 123). In obese and T2DM subjects, β-cell function is considerably improved by calorie or dietary restriction (124). However, the underlying molecular mechanisms mediating this beneficial effect remain unclear. Although it has previously been suggested that islet cell death might be stimulated by autophagy through a direct (autophagic cell death) or indirect pathway (apoptosis) (76), autophagy is crucial for normal function and survival of pancreatic β cells by maintaining a balance between the synthesis, degradation, and subsequent recycling of cellular products (125-127). Hence, it seems that modulated autophagy, but not excessive autophagy, could be a possible mechanism explaining the beneficial effects of CR on β-cell function.

To investigate this hypothesis, Gao et al. conducted a study in which four week old male mice were randomly divided into two groups to receive high-fat diet (HF) or normal chow (NC) for eight weeks (124). Then, they were randomly assigned into five groups: NC AL (ad libitum), NC CR, HF AL, HF → NC (HF switching to NC, ad libitum), HF → NC CR. The food intakes of both CR groups were 60 % of that of NC AL group. The intervention was for three weeks. A significant increase in the LC3II/LC3I ratio and beclin-1 level and a notable decrease in p62 was activated in NC CR, HF AL and HF → NC groups, though differences in autophagy levels amongst these groups were not significant. Since β-cell autophagy was

activated in both CR groups, and autophagy activity was closely related to the energy metabolism level, AMPKα phosphorylation was also evaluated; it was, however, only up-regulated in NC CR islets, the differences among the other four groups being non-significant. These findings indicate the positive impact of CR on β-cell function and insulin resistance, which is perhaps mediated by β-cell autophagy, and is independent of AMPK activation (124).

To assess the effect of various dietary interventions on islet cell autophagy, Sun et al assigned 14-16 month male rats into three groups: normal diet (ND), high fat diet (HFD) and calorie restricted diet (CRD) (128). After a 16 week intervention, islet cell autophagic markers, including LC3B and LAMP2, were significantly upregulated in both CRD and HFD groups compared with ND. In addition, the upregulation of acid phosphatase was significantly increased in CRD and HFD, while no significant differences were observed between the HFD and CRD groups (128).

The effect of fasting on the pancreatic acinar cells was investigated by Nevalainen et al (129). 2-3 month old mice were assigned to an AL diet (control group) or fasting groups in which animals were food-deprived for one, two or three days. The number and size of heterogenous dense bodies, mostly located in the Golgi areas, and autophagy activity was increased in response to fasting, as suggested by the localization of reaction product for acid phosphatase activity (129).

A summary of the studies evaluating the effect of fasting or calorie restriction on pancreas autophagy is shown in Table 6.

The effect of fasting or calorie restriction on autophagy in other tissues

The effect of nutrient deprivation on autophagy stimulation of white blood cells from mice or healthy human volunteers was assessed by Pietrocola et al (130). Six-week-old female mice were divided into two groups to receive standard diet or be fasted for 48 h. In addition, nine healthy human volunteers received a zero-calorie diet (with water, tea and coffee ad

libitum) for 4 consecutive days. A significant increase was observed in the number of $LC3B^+$ puncta per cell in response to the nutrient deprivation in all murine leukocytes, although $LC3B^+$ puncta per cell increased only in neutrophils from starved volunteers; this indicates that autophagy flux in circulating leukocytes from mice and in cultured neutrophils from fasted human volunteers was stimulated in response to the nutrient deprivation (130).

The effect of fasting and refeeding on structures of the intestinal villi and epithelial cells in White Leghorn hens was evaluated by Yamauchi et al (131). The birds were fasted for 12 h to 20 days or refed for one day after each fasting period. Findings of this study showed that large lysosomal autophagous vacuoles, including mitochondria and dense bodies, were increased in the epithelial cells of the proximal intestine of chickens after 20 days of fasting. However, the size of these vacuoles was reduced after only one day of refeeding. These results suggest that intracellular digestion may increase through lysosomal autophagy in response to fasting (131).

Using an *in vitro* approach, the effect of Cisplatin (CDDP), in association with fasting in wild type and mutated $BRAF^{V600E}$ melanoma cell lines, was investigated (132). CHL-1 and SK Mel 28 cells were subjected to starvation or exposed to CDDP for 2, 4 and 6 hours to evaluate autophagy flux, p62 protein levels and LC3 conversion (LC3-II). In response to nutrient deprivation, autophagy flux in CHL-1 was increased, although SK Mel 28 were resistant to nutrient shortage-induced autophagy. Moreover, no significant modulation of either p62 protein levels nor LC3-II accumulation was found in response to combined treatment of both melanoma cell lines with CDDP and food deprivation (132).

The effect of CR on autophagy induction was also evaluated in yeast (23). Autophagy was stimulated early during chronological aging in response to the water wash CR and low glucose CR, and the most persistent autophagy induction occurred under low glucose CR (23).

A summary of the above studies is shown in Table 7.

Conclusion

The studies reviewed here, while being heterogeneous in terms of interventional approaches, the species and ages of the experimental animals and the duration and intensity of intervention, overwhelmingly suggest that both fasting and CR have a salient role in upregulation of autophagy markers and autophagy activation. Modulated autophagy can be achieved by both fasting and CR, and plays a crucial role in normal function and hemostasis of cells, leading to an improvement in the health and function of various organs and tissues, muscle, liver, kidney, heart, pancreatic and the nervous system being examples. Whilst more studies are indicated to better understand the benefits, fasting and CR can be considered as a safe, practical and novel approach to improve cell health in all species, particularly mammals.

Figure 1. Overview of the effect of Fasting or Calorie restriction on major signaling pathways that contribute to autophagy induction in different organ and tissues. Food deprivation stimulates autophagy through different pathways, including upregulation of several proteins involved in autophagy such as Atg6, Atg7, Atg8, LC3-II, Beclin1, p62, Sirt1, LAMP2, ULK1 and ATG101. Number, size and signal of autophagosomes, autophagic vacuoles and dense bodies dramatically increase in response to the starvation. AMPK phosphorylation (autophagy inducer) and S6 dephosphorylation (indicating mTOR suppression) are the other pathways to promote autophagy in response to the starvation. mTOR phosphorylation which causes autophagy suppression, is reduced in response to fasting or CR. Likewise, the level of AKt protein (which activates mTOR) and phospho-S6RP (which indirectly indicates mTOR activity) are decreased in response to food deprivation.

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Figr-1

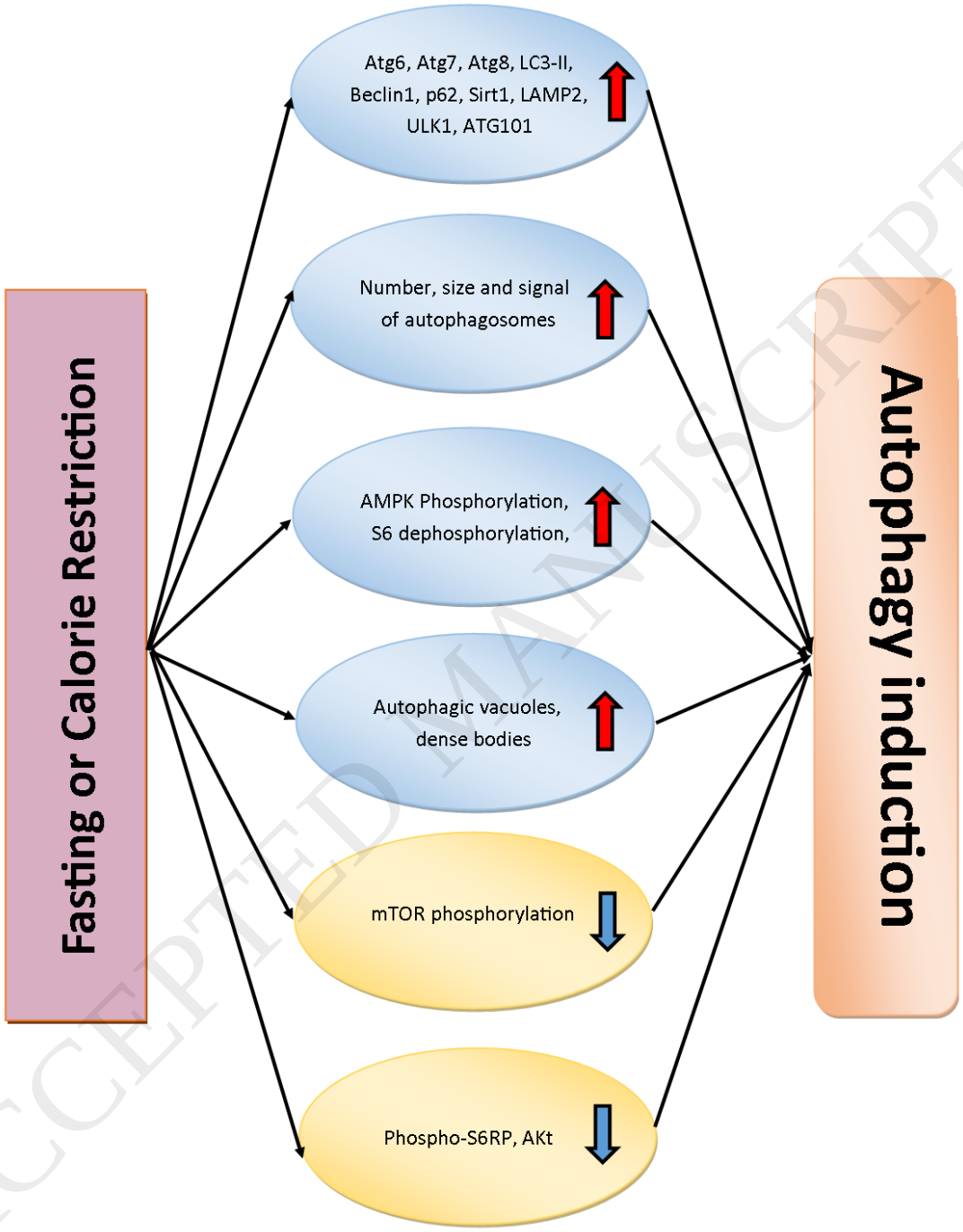

Table 1. The effect of fasting or calorie restriction on neuronal autophagy

First Author	Year	Intervention/ main component	Target tissue	Human/ animal	Main Results
Alirezaei, M 74	2010	Short-term fasting (24 or 48 hours)	Neuronal autophagy	Mice	Short-term fasting leads to a dramatic upregulation in neuronal autophagy. The increased neuronal autophagy is revealed by changes in autophagosome abundance and characteristics, and by diminished neuronal mTOR activity *in vivo*, demonstrated by a reduction in levels of phosphorylated S6 ribosomal protein in Purkinje cells.
Chen, X. (75)	2015	0 to 48 hours Fasting induced macroautophagy in neurons	Neurons	Mice	Fasting increased the number, size and signal intensity of autophagosomes in neurons. In Alzheimer's disease model mice, these parameters of autophagosome were higher at basal levels (before fasting) and increased more rapidly as a consequence of fasting than in control mice.
Madorsky, I. (76)	2009	Dietary restriction by intermittent fasting (IF) for five months	Peripheral nervous system	Mice	IF regimen increased expression of autophagy-associated proteins, Atg7 and LC3, and decreased steady state levels of p62, a protein that serves as a link between LC3 and ubiquitinated substrates, which indicates that autophagy was increased within the nerves of IF neuropathic mice.
Rangaraju, S. 77	2009	*In vitro*: Medium deprived of amino acids and serum (Stv medium) *In vivo*: Rats of four distinct ages (8, 18, 29 and 38 months) kept on an ad libitum (AL) or a 40% calorie restricted (CR) diet.	*In vitro:* Schwann cells from the sciatic nerves of young and old rats *In vivo*: myelinated peripheral nerves	Rat	*In vitro*: In cells from young rats, Atg7 and the conversion of LC3 I to LC3 II were increased. In cells from older rats, the response to nutrient deprivation is lessened, especially for Atg7. *In vivo*: lysosome-associated membrane protein 1 (LAMP1) and the autophagic protein Atg7 gradually increased with age. The CR intervention reduced the age-associated increase in the levels of pS6 and total S6.

Table 2. The effect of fasting or calorie restriction on liver autophagy

First Author	Year	Intervention/ main component	Target tissue	Human/ animal	Main Results
Donati, A. (85)	2013	Anti-aging caloric restriction (Alternate day fasting) for 10 month	Liver	Male Sprague-Dawley rats	Liver autophagy was maintained at very high levels throughout life in response to the anti-aging caloric restriction.
Kovács, A. L. (86)	1989	Food-deprivation, re-feeding and food deprivation (2, 12, 24, 48 and 72 hours)	Liver and exocrine pancreatic cells	Mice	In comparison to the feeding time, the cytoplasmic volume fraction of autophagic vacuoles significantly increased after 12, 24, 48 and 72 hours of fasting in both liver and exocrine pancreatic cells.
Krustew, L. P. (87)	1976	Fasting for 1-8 days	Liver lysosomes	Rat	Starvation stimulated autophagy. Autophagy is a fundamental process during starvation. In response to fasting, the number of lysosomes in hepatocytes rose.
Luévano-Martínez, L. A. (88)	2017	Calorie restriction (fed daily with 60% of a diet supplemented with micronutrients) for four months	Liver mitochondria	Rat	The LC3-II/LC3-I ratio was increased dramatically in the hepatocyte mitochondrial indicating enhanced autophagy.
Rickenbacher, A. (31)	2014	Fasting for one, two or three days prior to the ischemic insult	Liver enzymes and histology	Mice	The expression of LC3 II and beclin1 were increased indicating enhanced autophagy, together with a reduction in circulating high mobility group box 1 (HMGB1). Hepatic Sirt1 activity was upregulated after one day fasting.
Teckman, J. H. (89)	2002	18-h interval of fasting	Liver	PiZ mouse model	Although fasting induced a marked autophagic response in wild-type mice, the autophagic response was already activated in PiZ mice and did not further increase with fasting.
Wohlgemuth, S. E. (12)	2007	40 % calorie restriction Diet	Liver and heart	Fisher 344 rats	Autophagy was enhanced by calorie restriction in the heart. However, CR did not cause a substantial effect on the expression of autophagic proteins in the liver.

Wu, J. W. (90)	2012	5 and 48 hours fasting	WAT, liver, skeletal muscle, and heart	Mice	Levels of mRNA related to autophagy and proteolysis were increased in the white adipose tissue (WAT) of adipose-specific ATGL-deficient (ATGLAKO) mice, as well as in liver, skeletal muscle, and heart after 48 hours fasting.
Yamamoto, J. (93)	2015	1- or 2-day fasting	Various mouse tissues including: Liver, heart, skeletal muscle	Mouse	Post-fasting: The expression of LC3b was upregulated upon fasting in heart > skeletal muscle > thymus > lung, small intestine, testis, colon, and liver. p62 was upregulated in heart > skeletal muscle > thymus > colon, lung, kidney, spleen, and small intestine.
Chen, L. (97)	2017	12 or 24 hours fasting	Hepatocyte	Mice	In response to fasting, hormone fibroblast growth factor 21 (FGF21) levels as well as autophagy (reflected by increased LC3-II and decreased P62 levels) were found to be increased in the liver. After 24 hours of fasting, autophagy was increased in the hepatocytes in wild type mice. However, after 24 hours fasting, much higher levels of LC3-II and P62 were found in Fgf21−/− mice compared with WT animals.
Derous, D. (99)	2017	Calorie restriction (CR) (0% to 40% CR) for three months	*Hepatic transcripts of male C57BL/6 mice*	Mice	Graded CR had a positive effect on autophagy. A significant increase was observed in autophagy levels with increasing levels of CR.

Table 3. The effect of fasting or calorie restriction on heart autophagy

First Author	Year	Intervention/ main component	Target tissue	Human/ animal	Main Results
Dutta, D. (111)	2014	Short-term moderate calorie restriction (20 %), either alone or in combination with resveratrol for six weeks	Heart	Fischer rats	Autophagy was not induced in response to 20% CR or resveratrol alone for 6 weeks, but 20% CR in combination with 50 mg/kg/day resveratrol resulted in an induction of autophagy in the hearts of 26-month-old rats.
Makino, N. (113)	2015	calorie restriction (OLETF-CR: 30 % energy reduction compared to normal diet) for 32 weeks	Cardiac telomere biology	Diabetic rats	The LC3-II/LC3-I ratio was notably increased in the heart and liver of CR diabetic rats compared with AL diabetic animals. No significant change was found in the LC3-II/LC3-I ratio in the heart and liver of CR animals compared with AL non-diabetic rats. No significant difference was observed in the expression levels of beclin 1 among all the study groups
Andres, A.M. (114)	2016	1. Diet induced obesity (DIO)-2. Fasting. Ad Libitum with normal chow (13% kcal/100 kcal fat) or a lard-based high-fat diet (fat 60% kcal/100 kcal fat) for 4–20 weeks. Then, overnight fasting.	Heart	Obese mice	Fasting induces autophagy in the hearts of lean mice, though hearts of obese mice exhibited impaired autophagy, altered proteome, and a discordant response to nutrient deprivation.
Godar, R. J. (115)	2015	Intermittent fasting for 6 weeks	Myocardial ischemia-Reperfusion injury	Mice	No significant change was found in autophagosome abundance after 24 hours fasting. A dramatic increase was observed after 48 hours fasting. Findings indicated that cardiomyocyte autophagy was induced by each episode of fasting, then returned to basal levels on the fed days.
Makino, N. (116)	2016	30 % calorie restriction for 20 weeks	Heart and liver	Mice	At post intervention, a significant increase was found in the LC3-II/LC3-I ratio, while the expression of p62 was significantly decreased in the heart tissue of CR wild type mice.

					No changes occurred in the LC3-II/LC3-I ratio or the expression of p62 in FoxO1-knockout heterozygous mice ($FoxO1^{+}/^{-}$).

Table 4. The effect of fasting or calorie restriction on Muscle autophagy

First Author	Year	Intervention/ main component	Target tissue	Human/ animal	Main Results
Ogata, T. (125)	2010	Fasting for 1, 2, or 3 days	Fast-twitch skeletal muscle	Fischer-344 rats	Preferential atrophy of fast-twitch muscle is associated with induction of autophagy during fasting and differences in autophagy regulation are attributable to differential signal regulation in soleus and plantaris muscle
Qi, Z. (126)	2014	Short-term Fasting (24 hours)	Skeletal muscle	Mice	Fasting significantly increases the mRNA level of several autophagy markers and LC3 lipidation. N-Acetylcysteine (NAC) supplementation significantly reduced mRNA expression of Atg6, Atg7, Atg8 and Atg9 in skeletal muscle. Except for Atg9, the Atgs were upregulated in response to fasting.
Rittig, N. (127)	2017	36 hours fasting before the following trials: Whey protein beverage (LWH) with isocaloric carbohydrate-(CHO), soy protein (SOY), and soy protein +3 g HMB (HMB) during fasting-induced catabolic conditions.	Muscle protein	Human	The LC3II/LC3I ratio was decreased during LWH and SOY intake compared with the fasting period. In comparison to the fasting period, during the sipping period phosphorylation of mTOR, Akt (the activator of mTOR, mediating autophagy inhibition) as well as the downstream target of mTOR, 4E binding protein 1 (4EBP1), were elevated in all 4 groups.
Vendelbo, M. H. (128)	2014	72-hour-fast	Skeletal muscle protein metabolism	Human	mTOR phosphorylation was reduced by about 40% in response to fasting. Fasting increased expression of the autophagic marker LC3B-II by ∼30%. p62 is degraded during autophagy but was increased by ∼10% during fasting.
Wohlgemuth, S. E. (129)	2010	Mild, life-long calorie restriction (8%) alone or combined with life-long voluntary exercise.	Muscle	Fischer 344 rats	Mild CR attenuates the age-related impairment of autophagy in rodent skeletal muscle.
Yang, L. (130)	2016	∼30% CR diets for 3-15 years	Skeletal muscle	Human	Autophagy genes, including ULK1, ATG101, beclin-1, APG12, LC3, GAPRAP/GATE-16, and autophagin-1, were significantly upregulated in response to CR.

					CR in humans is associated with sustained rises in serum cortisol, reduced inflammation, and increases in key molecular chaperones and autophagic mediators involved in cellular protein quality control and removal of dysfunctional proteins and organelles

Table 5. The effect of fasting or calorie restriction on kidney autophagy

First Author	Year	Intervention/ main component	Target tissue	Human/ animal	Main Results
Kume, S. (133)	2010	40% calorie restriction compared with normal for 12 months	Mitochondrial autophagy - aged kidney	Mice	Adult-onset and long-term CR in mice promoted increased Sirt1 expression in aged kidney. Higher levels of sequestosome 1 (Sqstm1), (a marker for in vivo impaired autophagy) were found in the kidney of AL mice. The ratio of LC3I to LC3II conversion and LC3 dots were higher in the CR mice.
Ning, Y. C. (138)	2013	40% calorie restriction for 8 weeks	Renal	Sprague-Dawley rats	Short-term CR increased autophagy flux (expression of the LC3-II/LC3-I and Beclin-1) and subsequently reduced oxidative damage.

Table 6. The effect of fasting or calorie restriction on Pancreas autophagy

First Author	Year	Intervention/ main component	Target tissue	Human/ animal	Main Results
Gao, X. (143)	2015	Moderate (40 %) calorie restrction in diet-induced obese (DIO) mice for three weeks	β-cell function	Mice	Moderate CR to achieve normal weight reversed β-cell dysfunction and insulin resistance, and restored glucose homeostasis in DIO mice. The up-regulation of β-cell autophagy may play a role in this process, independent of AMPK activation.
Sun, Q. (147)	2016	A normal diet (ND), a high-fat diet (HFD), or a calorie-restricted diet (CRD, containing 50% of the calorie content of the ND) for 16 weeks	Pancreatic tissue	Sprague Dawley rats	Islet cell autophagic markers, including LC3B and LAMP2, were significantly upregulated in both CRD and HFD groups compared with ND. Acid phosphatase upregulation was significantly increased in CRD and HFD groups.
Nevalainen, T. J. (148)	1974	Fasting for 1, 2 or 3 days	Pancreas	Mouse	The number and size of heterogenous dense bodies, mostly located in the Golgi areas, and autophagy activity was increased in response to fasting.

Table 7. The effect of fasting or calorie restriction on autophagy of blood cells, intestinal villi and epithelial cells, melanoma and yeast

First Author	Year	Intervention/ main component	Target tissue	Human/ animal	Main Results
Pietrocola, F. (150)	2017	Fasting of mice for 48 h (which causes ∼20% weight loss) or starvation of human volunteers for up to 4 d (which causes <2% weight loss)	Blood cells	Mice and human	Whereas all murine leukocyte subpopulations significantly increased the number of LC3B+ puncta per cell in response to nutrient deprivation, only neutrophils from starved volunteers showed signs of activated autophagy.
Yamauchi, K. (151)	1996	Fasting for 12 hours to 20 days or refeeding for one day after each fasting period	Villi and epithelial cells in each part of the small intestine	Hens	After for 20 days fasting, large lysosomal autophagous vacuoles including mitochondria and dense bodies were observed in the epithelial cells of the proximal intestine. These were reduced in size by refeeding for only one day.
Antunes, F (152)	2017	Anti-cancer drugs (Cisplatin (CDDP)) with calorie restriction.	Melanoma	*In vitro:* Wild type and mutated $BRAF^{V600E}$ melanoma cell lines	In response to nutrient deprivation, autophagy flux in CHL-1 was increased, although SK Mel 28 were resistant to nutrient shortage-induced autophagy. No significant modulation of either p62 protein levels nor LC3-II accumulation was found in response to combined treatment of both melanoma cell lines with CDDP and food deprivation.
Aris, J.P (29)	2013	Chronological life span (CLS) by calorie restriction (CR), water wash CR and low glucose CR	Budding yeast Saccharomyces cerevisiae	*In vitro:* Yeast	Autophagy was induced during chronological aging in response to the water wash CR and low glucose CR.